L Number	Hits	Search Text	DB	Time stamp
1	1831	transgenic WITH pig	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/06/13 13:55
7	502	(transgenic WITH pig) and globin	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/06/13 13:55
13	23	((transgenic WITH pig) and globin) and zeta	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/06/13 13:56
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-	19	Shen NEAR james	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/06/11 14:13
-	7	HS-40 ADJ enhancer	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/06/11 14:13
-	34	zeta ADJ globin	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/06/11 14:13
-	16	(zeta ADJ globin) and transgenic	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/06/11 14:13
-	6	TCTGAGTCA	USPAT; US-PGPUB; EPO; JPO; DERWENT	2003/06/11 14:13
-	7	(US-6451334-$ or US-6022738-$ or US-6172039-$ or US-5919997-$ or US-5827693-$ or US-4822821-$ or US-6303845-$).did. or (US-20020133838-$ or US-20020148000-$ or US-20020108134-$).did. or (US-6303845-$).did.	USPAT; US-PGPUB; DERWENT	2003/06/11 14:13

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Shen

(10) **Patent No.: US 6,303,845 B1**
(45) **Date of Patent: Oct. 16, 2001**

(54) **HS-40 ENHANCER-CONTAINING VECTOR**

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(52) **U.S. Cl.** **800/18**; 800/8; 435/320.1; 435/325; 536/23.1; 536/24.5

(58) **Field of Search** 800/8, 18; 536/23.1, 536/24.1; 435/320.1, 325

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(57) **ABSTRACT**

The invention relates to a transgenic animal whose somatic and germ line genomic DNA includes at least one copy of a transgene having (1) a transcriptional start site; (2) a promoter operably linked to the transcriptional start site; and (3) an enhancer operably linked to the promoter, the enhancer including the nucleotide sequence of SEQ ID NO:1, where the transgenic animal expresses a transcript driven by the promoter, the level of expression in at least one cell type of the animal being positively correlated with the copy number of the transgene.

14 Claims, No Drawings

HS-40 ENHANCER-CONTAINING VECTOR

This application is a divisional of U.S. Ser. No. 09/205,015, filed Dec. 4, 1998, now abandoned.

BACKGROUND OF THE INVENTION

HS-40 is a 350–400 bp enhancer element located about 40 kb upstream of ζ-globin gene, which is expressed in the human embryonic erythroblasts but not in the human adult erythroblasts. Specific elements within the HS-40 enhancer have been identified, including GATA-1 motifs, NF-E2/AP1 motifs (a 3' and a 5' motif), and a Sp1 binding site.

SUMMARY OF THE INVENTION

The invention is based on the discovery that a single nucleotide change in the 3'NF-E2/AP1 element of the human HS-40 enhancer, unlike the wild type HS-40 enhancer, confers position-independent and copy number-dependent expression on a transgene. In addition, the single nucleotide change allows expression of the gene in the cells of an adult mouse, an effect not seen for the wild type HS-40 enhancer.

Accordingly, the invention features a viral expression vector (e.g., a retrovirus) having a nucleic acid including (1) a transcriptional start site; (2) a promoter (e.g., a tissue-specific promoter such as a ζ-globin promoter) operably linked to the transcriptional start site; and (3) an enhancer operably linked to the promoter, the enhancer including the mutated NF-E2/AP1 (mtNF-E2/AP1) DNA sequence TCTGAGTCA (SEQ ID NO:1) or the RNA equivalent thereof The underlined "T" represents a mutation of the wild type "G" in the wild type NF-E2/AP1 (wtNF-E2/AP1) sequence. In a specific embodiment, the enhancer includes the minimal mutated HS-40 DNA sequence

AGATAACTGGGCCAACCATGACTCAGTGCTTCTGGAGGCCAACAGGACTTCT GAGTCATCCTGTGGGGGTGGAGGTGGGACAAGGGAAAGGGGTGAATGGTAC TGCTGATTACAACCTCTGGTGCTGCCTCCCCCTCCTGTTTATCT (SEQ ID NO:2)

or an RNA equivalent thereof. The bold sequence represents the mtNF-E2/AP1 site with the G to T mutation underlined. The minimal HS-40 enhancer sequence excludes a 5' GATA1(b) site because it has been shown that this site is not necessary for HS-40 enhancer activity (Zhang et al., J Biol Chem 270:8501–8505, 1995).

The enhancer can also include the fall mutated HS-40 enhancer sequence:

TCGACCCTCTGGAACCTATCAGGGACCACAGTCAGCCAGGCAAGCACATCTG CCCAAGCCAAGGGTGGAGGCATGCAGCTGTGGGGGTCTGTGAAAACACTTGA GGGAGCAGATAACTGGGCCAACCATGACTCAGTGCTTCTGGAGGCCAACAGG ACTTCTGAGTCATCCTGTOGGOGTGGAOGTGGGACAAGGGAAAGGGGTGAA TGGTACTGCTGATTACAACCTCTGGTGCTGCCTCCCCCTCCTGTTTATCTGAG AGGGAAGGCCATGCCCAAAGTGTTCACAGCCAGGCTTCAGGGGCAAAGCCT GACCCAGACAGTAAATACGTTCTTCATCTGGAGCTGAAGAAATTC (SEQ ID NO:3)

or an RNA equivalent thereof The bold sequence represents the mtnf-E2/AP1 site with the G to T mutation underlined. This sequence is referred to herein as the mtHS-40 sequence, which differs from the wild type HS-40 (wtHS-40) sequence by the G/T mutation indicated above. Again, the single mutation is underlined. The vector can also contain a transcriptional termination signal (e.g., a polyadenylation signal). In other embodiments, the promoter drives transcription of a MRNA encoding a polypeptide (e.g., a growth hormone), the transcription beginning from the transcriptional start site.

A promoter is a nucleotide sequence required to facilitate transcription from a transcriptional start site, which is the site at which the first nucleotide of the transcript is transcribed, the nucleotide being complementary to the corresponding nucleotide in the nucleic acid. A promoter operably linked to a transcriptional start site means that the promoter is capable of driving transcription from the transcriptional start site in the absence of farther nucleotide sequences.

An enhancer is a nucleic acid sequence which increases the level of transcription from a promoter. Enhancers need not be in any specified position in the nucleic acid in relation to the promoter, transcriptional start site, or transcriptional termination site. All that is required for a specific enhancer to be operably linked to a specific promoter is that the presence of the enhancer increases transcription driven by that promoter.

A transcriptional termination signal is a nucleic acid sequence which terminates transcription of a transcript. A variety of promoters, enhancers, and transcriptional termination signals are known in the art.

A viral expression vector is any combination of a nucleic acid and at least one protein which is useful for delivering a nucleic acid into a cell so as to express a transcript encoded by the nucleic acid in the cell. Other components, such as a lipid bilayer can also be present in the vector. An example of a viral expression vector is a retrovirus.

The invention also includes a transgenic animal (e.g., a mouse or other rodent, pig, rat, cow, chicken, turkey, or sheep) whose somatic and germ line cells contain at least one copy of a transgene comprising (1) a transcriptional start site; (2) a promoter (e.g., a tissue-specific promoter such as a ζ-globin promoter) operably linked to the open reading frame; and (3) an enhancer operably linked to the promoter. The enhancer includes the nucleotide sequence of SEQ ID NO:1 (e.g., SEQ ID NO:2). The transgenic animal expresses a transcript driven by the promoter, where the level of expression in at least one cell type (e.g., a erythroblast) of the animal is proportionally dependent on the copy number of the transgene, i.e., the greater the copy number, the greater the expression. Such a transcript can be a mRNA encoding a polypeptide (e.g., a growth hormone). In other embodiments, the somatic and germ line cells contain more than 5 copies (e.g., more than 15 copies) of the transgene.

The invention also features a method of expressing a transcript in an animal (e.g., a mouse, pig, rat, cow, chicken, turkey, or sheep) by administering to the animal a nucleic acid comprising (1) an transcriptional start site for the transcript; (2) a promoter (e.g., a tissue-specific promoter such as a ζ-globin promoter) operably linked to the transcriptional start site; and (3) an enhancer operably linked to the promoter, the enhancer comprising the DNA sequence of SEQ ID NO:1 or 2 or the RNA equivalent thereof. The transcript can be a mRNA encoding a polypeptide. The nucleic acid can be administered by parenteral injection (e.g., intramuscular injection) or via a viral expression vector. The nucleic acid can further include a transcriptional termination signal (e.g., a polyadenylation signal).

Nucleic acids and viral vectors containing an enhancer having the mtNF-E2/AP1 sequence described above can be used to express a therapeutic antisense RNA or mRNA

encoding a therapeutic polypeptide in an animal in a position-independent and transgene copy number dependent manner. This was an unexpected result because, previously, transgene expression was limited by position-effect variegation, silencing of transgenes, and the inability to increase expression by increasing the copy number of the transgene. See, e.g., Sabl et al., Genetics 142:447–458, 1996; Palmer et al., Sharpe et al., EMBO J 11:4565–4572, 1992; and Chen et al., Proc Natl Acad Sci USA 94:5798–5803, 1997. By inclusion of an enhancer containing the mtNF-E2/AP1 sequence in the transgene sequence, these deficiencies in transgene expression are removed. Enhancement of transgene expression can result in transgenic animal models exhibiting more severe symptoms so that therapeutic efficacy in those models can be measured in a wider range of symptom severity. Examples of such models, which can be improved by the present invention, are described in U.S. Pat. Nos. 5,811,634 and 5,675,060

Other features or advantages of the present invention will be apparent from the following detailed description and also from the claims.

DETAILED DESCRIPTION

The invention relates to nucleic acids and viral vectors containing an enhancer with a mutated NF-E2/AP1 site (e.g., the mtHS40 enhancer), and their use in expressing RNA in an animal. Nucleic acids including the mtNF-E2/AP1 site can be used to form transgenic animals of the invention which express an antisense transcript or a mRNA encoding the protein to be expressed in the transgenic animal. The expression of the transgene is not affected by its position in the genome, nor is the expression inhibited at high transgene copy numbers (e.g., above 5, 7, 9, 14, or 19 copies). Instead, the expression level is directly correlated with transgene copy number, thereby allowing high levels of expression at high transgene copy numbers.

Introduction of a transgene into the fertilized egg of an animal (e.g., a mammal) is accomplished by any number of standard techniques in transgenic technology. See, e.g., Hogan et al., Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986. Most commonly, the transgene is introduced into the embryo by way of microinjection.

Once the transgene is introduced into the egg, the egg is incubated for a short period of time and is then transferred into a pseudopregnant animal of the same species from which the egg was obtained (Hogan et al., supra). In the case of mammals, typically 125 eggs are injected per experiment, approximately two-thirds of which will survive the procedure. Twenty viable eggs are transferred into pseudopregnant mammal, four to ten of which will develop into live pups. Typically, 10–30% of the pups (in the case of mice) carry the transgene.

To identify the transgenic animals of the invention, progeny are examined for the presence of the transgene using standard procedures such as Southern blot hybridization or PCR. Expression of the transgene can also be assessed using Northern blots, Western blots, and immunological assays.

Without further elaboration, it is believed that one skilled in the art can, based on the above disclosure and the description below, utilize the present invention to its fullest extent. The following example is to be construed as merely illustrative of how one skilled in the art can practice the invention and are not limitative of the remainder of the disclosure in any way. Any publications cited in this disclosure are hereby incorporated by reference.

Production of Transgenic Mice

Transgenic mice were produced by microinjection of DNA fragments into the pronuclei of fertilized mouse eggs as described in Brinster et al., Cell 27:223–231, 1981 and Costantini et al., Nature 294:92–94, 1981. Plasmids pHS40-ζ597-GH and pHS40(r-mt 1)-ζ597-GH are described in Zhang et al., Mol Cell Biol 13:2298–2308, 1993. Digestion of these plasmids with EcoRI, NdeI, and ScaI yielded 3.12 kb DNA fragments containing the HS-40 enhancer, the ζ-globin promoter, and the growth hormone (GH) open reading frame. The 3.12 kb DNA fragments were eluted from soft agarose gels, purified, and used for microinjection.

Transgenic founders were identified and their transgene copy number determined by Southern blot analysis of tail DNA. The founders were then bred with nontransgenic C57/B6 mice to establish lines. The morning on which the copulatory pluf was observed was designated 0.5 day postcoital. For analysis of fetal (14.5 days postcoital) and 5 embryonic (9.5 days postcoital) mice, transgenic males were mated to nontransgenic C57/B6 females. Transgenic pups were identified by PCR analysis of fetal mice tails or of embryo DNA. For each identification, duplicate PCR reactions were carried out using one 5' primer from the ζ-globin promoter region, and two different 3' primers from the GH region (see below).

A total of 9 founders with wild type HS40-ζGH (wt) and 10 founders with the mutant HS40-ζGH (mt) have been obtained. The copy numbers of integrated fragments in wtHS-40-containing mice vary from 1 to more than 100, as shown in Table 1.

TABLE 1

Mutant HS-40 Transgene			Wild Type HS-40 Transgene		
Founder line	Copy number	hGH, ng/ml	Founder line	Copy number	hGH, ng/ml
1A*	1	470	1A*	1	36
1B*	1	530	1B*	1	20
1C*	1	1,060	2	2	14
2	2	650	3	3	22
3	3	1,260	5	5	5
8*	8	2,990	10*	10	13
10*	10	3,360	13*	13	187
13*	13	4,650	100A	>100	1,400
15*	15	5,560	100B	>100	30
19*	19	6,490			

In Table 1, the founders for which lines have been established are indicated by an asterisk. Mice with the wtHS-40 transgene were assayed at the age of 5 months except founder 1B, which was evaluated at 9 months old. Mice with the mtHS-40 transgene were assayed at the age of 4 months except founder 15, which was evaluated at 2 months old.

The ζ-globin promoter activities in the founder mice were first measured with a blood GH assay as described in Zhang et al., supra. The levels of human GH in the blood were quantitated with the Allegro hGH radioimmunoassay kit from Nichols Institute. When the concentration of GH in the blood exceeded 50 ng/ml, the samples were first diluted with horse serum in order perform the assay in a linear range.

It was known that the amount of secreted enzyme molecules are good representations of the quantities of mRNAs inside the expressing cells (Zhang et al., supra; Palmiter et al., Nature 300:611–615, 1982; Palmiter et al., Science 222:809–814, 1983; Hammer et al., Nature 315:680–683, 1985; and Selden et al., Mol Cell Biol 6:3137–3179, 1986). The level of GH in wtHS-40 transgenic mice were all low

and comparable to non-transgenic controls. This was consistent with observations that the human ζ-globin promoter activity is essentially shut off in adult transgenic mice, even when it is linked in cis with the wtHS-40 enhancer or with the β-globin locus control region (Pondel et al., Nucl Acids Res 20:5655–5660, 1992; Robertson et al., Proc Natl Acad Sci USA 92:5371–5375, 1995; Albitar et al., Mol Cell Biol 11:3786–3794, 1991; and Spanger et al., Nucl Acids Res 18:7093–7097, 1990).

In contrast, the blood GH levels of the ten founder mice having the mtHS-40 enhancer exhibited a roughly linear, positive relationship relative to transgene copy number. Further, the expression of the mtHS-40 transgene was integration site-independent (i.e., position-independent) because the integration sites here were believed to be random and mice having similar transgene copy numbers exhibit similar level of expression. The blood GH levels in these founders at other ages, as well as these founders' progeny, were similar to the levels of expression in mtHS-40-containing mice, as shown in Table 1.

To analyze the GH RNA levels in transgenic fetuses and embryos, liquid N2-frozen embryos, fetuses, or fetal livers were manually homogenized, and the RNA isolated by standard acid guanidinium isothiocyanate-phenol-chloroform extraction (Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Plainview, N.Y., 2nd ed., 1989). For adult samples, the mice were rendered anemic by three injections of phenylhydrazine (40 μg/g of body weight) so that erythroblasts would enter the adult blood and be collected for analysis. The second injection was 8 hours after the first injection, and the third injection was 24 hours after the first. Six days after the first injection, the mice were sacrificed, and the RNA was isolated from different tissues. In all cases, the total RNA was used for the following assay without further purification.

RT-PCR was carried out as described in Chelly et al., Nature 333:858–860, 1988 and Foley et al., Trends Genet 9:380–385, 1993. Each reverse transcription reaction mixture contained 1 μg of RNA, 200 units of SUPERSCRIPT II® reverse transcriptase (Gibco BRL), and 20 mM oligo $d(T)_{15}$. One-twentieth of the cDNA was then amplified by PCR using Taq polymerase (Gibco BRL) and primers specific for human GH, mouse β^{major}, mouse ζ-globin promoter, or mouse G3PDH. Amplifications were carried out in a HYBRID OmniGene system with the following temperature profiles: an initial denaturation at 95° C. for 3 min, 53° C. for 1 min, and 72° C. for 1 min; followed by repeating cycles of 95° C. for 1 min, 53° C. for 1 min, and 72° C. for 1 min; and finally an elongation step at 72° C. for 5 min. Each PCR analysis was done in duplicate. The sequences of PCR primers used are as follows. For mG3PDH, TGAAGGTCGGTGTGAACGGATTTGOC (SEQ ID NO:4) was used as the 5' primer, and CATGTAGGCCATGAGGTCCACCAC (SEQ ID NO:5) was used at the 3' primer. For the human GH gene, GTCCCTGCTCCTGGCTTT (SEQ ID NO:6) was used as the 5' primer, and ATGCGGAGCAGCTCCAGGTT (SEQ ID NO:7) was used as the 3' primer. Another 3' primer used for the human GH gene was CATCAGCGTTTGGATGCCTT (SEQ ID NO:8). For the mouse β^{major} sequence, TGGGCAGGCTGCTGGTTA (SEQ ID NO:9) was used at the 5' primer, and TTAGTGGTACTTGTGAGCCAA (SEQ ID NO:10) was used as the 3' primer. For the mouse ζ-globin promoter sequence, CTGATGAAGAATGAGAGAGC (SEQ ID NO:11) was used as the 5' primer, and TAGAGGTACTTCTCATCAGTCAG (SEQ ID NO:12) was used as the 3' primer. The PCR product lengths were 980 bp for mouse G3PDH, 335 bp for mouse β^{major}, and 290 bp or 450 bp for ζ-GH. One-fifth of each PCR reaction was resolved on a 1.5% agarose-ethidium bromide gel, which was then documented using a IS1000 Digital Imaging System and saved as a TIF computer file. The band intensities were quantitated by the PhosphorImage System.

For semi-quantitative purposes, mouse G3PDH was used as the internal standard. The linearity of amplification of the G3PDH cDNA was first defined by amplification of serial dilutions of the cDNA samples. Twenty five cycles were chosen for amplifying mouse G3PDH since, under the reaction conditions described above, the signals were linear over a wide range of dilutions of cDNA. In the initial calibration test, G3PDH bands with similar intensities were obtained from the different tissue cDNA when the same amount of RNA was used for reverse transcription. The appropriate PCR cycle number used to amplify the human GH, mouse β^{major}, and mouse ζ-globin transcripts were 28, 25 and 28, respectively. The amount of different cDNA used for amplification were first determined by PCR using the mouse G3PDG primers, then individual PCR reactions were performed using the human GH, mouse β^{majo}r, or mouse ζ-globin primers.

It was known that, in the developing mouse, the first site of erythropoiesis is at the yolk sac blood island at 8–14 days of gestation. The major site of erythropoiesis then shifts to the fetal liver, and finally to the spleen at birth. The expression of GH transcripts from the mouse ζ-globin promoter in adult transgenic mice containing the mtHS-40 enhancer was examined. In all adult mice having the mtHS-40 transgene, the expression of GH RNA was restricted to the eiythroid tissues. Expression was roughly limited to the spleen and blood, with no expression in the liver or brain. Expression could not be detected in the blood of mice containing the mtHS-40 transgene unless the mice were first rendered anemic, indicating that expression was erytliroblasts-specific. Mice having the wtHS-40 transgene exhibited little, if any, expression.

The expression of the transgenic mice at the fetal stage also appeared to be erythroid-specific. ζ-GH transcripts could be detected in 14.5 day fetuses from transgenic mice with either mtHS-40 or wtHS-40 sequences. No ζ-GH transcripts were detected in non-transgenic control mice. A high intensity RT-PCR band was apparent in the reaction containing fetal liver RNA, consistent with the erythroid fetal liver being the major site of transcription of ζ-GH transgenes.

Changes in ζ-GH transgene expression were followed by RT-PCR. Transgenic mice having the wtHS-40 transgene exhibited the expected temporal pattern of expression during development, the level of ζ-GH transcripts was relatively high at the 9.5 day embryo stage but dropped significantly in the adult blood. In contrast, the transgenic mice having the mtHS-40 enhancer continued to express the ζ-GH transcript into adulthood. In addition, even with only one copy of the transgene, mice having the mtHS-40 expressed at a higher level than mice having the wtHS-40 enhancer, regardless of the stage of development.

These data indicated that the mtHS-40 enhancer sequence not only relieved the repression of the ζ-globin promoter in adulthood, but enhanced expression at all stages of development, even at one transgene copy per genome. When combined with the linear relationship between transgene copy number and expression level, as described above, the results indicated that mtHS-40 can be used as an enhancer of gene expression in a variety of contexts.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with he detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are also within the scope of this invention. For example, inconsequential deletions, additions, or substitutions of nucleotides within SEQ ID NOs: 1, 2, or 3 (i.e., do not affect the advantageous properties of the mtHS-40 enhancer) are within the scope of the claims.

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gccaaccatg actcagtgct tctggaggcc aacaggactt ctgagtcatc ctgtgggggt 180

ggaggtggga caagggaaag gggtgaatgg tactgctgat tacaacctct ggtgctgcct 240

ccccctcctg tttatctgag agggaaggcc atgcccaaag tgttcacagc caggcttcag 300

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<213> ORGANISM: Mus musculus

<400> SEQUENCE: 11

ctgatgaaga atgagagagc 20

<210> SEQ ID NO 12
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 12

tagaggtact tctcatcagt cag 23

What is claimed is:

1. A transgenic mouse whose somatic and germ line genomic DNA comptises at least one copy of a transgene comprising (1) a nucleic acid sequence encoding a polypeptide; (2) a ζ-globin promoter operably linked to the nucleic acid sequence; and (3) an enhancer operably linked to the promoter, the enhancer comprising the nucleotide sequence of SEQ ID NO:1,

wherein the transgenic mouse expresses the polypeptide, the level of expression in the erythroid cells of the mouse being correlated with the copy number of the transgene.

2. The transgertic mouse of claim 1, wherein the DNA contains more than 5 copies of the transgene.

3. The transgenic mouse of claim 2, wherein the DNA contains more than 15 copies of the transgene.

4. The transgenic mouse of claim 1, wherein the erythroid cells include a erythroblast.

5. The transgenic mouse of claim 1, wherein the enhancer comprises SEQ ID NO:2.

6. The transgenic mouse of claim 5, wherein the enhancer comprises SEQ ID NO:3.

7. The transgenic mouse of claim 1, wherein the expression of the transgene is independent of its position in the genomic DNA.

8. The transgenic mouse of claim 1, wherein the transgene is expressed in a cell of an adult form of the transgenic mouse.

9. An isolated cell whose genonic DNA comprises at least one copy of a transgene comprising (1) a nucleic acid sucnce encoding a polypeptide; (2) a ζ-globin promoter operably linked to the nucleic acid sequence; and (3) an enhancer operably linked to the promoter, the enhancer comprising the nucleotide sequence of SEQ ID NO:1,

wherein the cell expresses the polypeptide, the level of expression being correlated with the copy number of the transgene.

10. The cell of claim 9, wherein the DNA contains more than 5 copies of the transgene.

11. The cell of claim 10, wherein the DNA contains more than 15 copies of the transgene.

12. The cell of claim 9, wherein the enhancer comprises SEQ ID NO:2.

13. The cell of claim 12, wherein the enhancer comprises SEQ ID NO:3.

14. The cell of claim 9, wherein the expression of the transgene is independent of its position in the genomnic DNA.

* * * * *

FILE 'HOME' ENTERED AT 13:58:43 ON 13 JUN 2003)

FILE 'MEDLINE, AGRICOLA, CANCERLIT, SCISEARCH, CAPLUS, MEDICONF' ENTERED AT 13:59:23 ON 13 JUN 2003

L1 774 S ZETA(S)GLOBIN
L2 162 S L1 AND TRANSGENIC
L3 0 S L2 AND PIG
L4 1 S L1 AND PIG
L5 5 S L2 AND (HS-40 ENHANCER)
L6 5 DUP REM L5 (0 DUPLICATES REMOVED)
L7 5 SORT L6 PY

=> d an ti so au ab pi l7 5 1

L7 ANSWER 5 OF 5 CAPLUS COPYRIGHT 2003 ACS
AN 2001:757858 CAPLUS
DN 135:314417
TI Vectors containing mutated **HS-40 enhancer** of .**zeta**.-**globin** gene promoter and its regulation of transgene expression in **transgenic** mice
SO U.S., 7 pp., Division of U.S. Ser. No. 205,015, abandoned.
CODEN: USXXAM
IN Shen, Che-Kun James
AB The invention relates to a mutated **HS-40 enhancer** of .**zeta**.-**globin** gene promoter, a 350-400 bp enhancer element located about 40 kb upstream of .**zeta**.-**globin** gene. HS-40 is the major cis-acting regulatory element responsible for the developmental stage-and erythroid lineage-specific expression of the human .alpha.-like **globin** genes, the embryonic .**zeta**. and the adult .alpha.2/.alpha./1. The invention is based on the discovery that a single nucleotide change in the 3'NF-E2/AP1 element of the human **HS-40 enhancer**, unlike the wild type **HS-40 enhancer**, confers position-independent and copy no.-dependent expression on a transgene. In addn., the single nucleotide change allows expression of the gene in the cells of an adult mouse, an effect not seen for the wild type **HS-40 enhancer**. Accordingly, the invention provides a viral expression vector (e.g., a retrovirus) expressing a transgene regulated by (1) a transcriptional start site; (2) a promoter (e.g., a tissue-specific promoter such as .**zeta**.-**globin** promoter) operably linked to the transcriptional start site; and (3) the above mutated **HS-40 enhancer** operably linked to the promoter.

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
US 6303845	B1	20011016	US 2000-536094	20000324
US 2002108134	A1	20020808	US 2001-977432	20011015

L7 ANSWER 1 OF 5 SCISEARCH COPYRIGHT 2003 THOMSON ISI
AN 93:191049 SCISEARCH
TI TRANSCRIPTIONAL ACTIVATION OF HUMAN **ZETA**-2 **GLOBIN** PROMOTER BY THE ALPHA-**GLOBIN** REGULATORY ELEMENT (HS-40) - FUNCTIONAL-ROLE OF SPECIFIC NUCLEAR FACTOR-DNA COMPLEXES
SO MOLECULAR AND CELLULAR BIOLOGY, (APR 1993) Vol. 13, No. 4, pp. 2298-2308. ISSN: 0270-7306.
AU ZHANG Q Y; REDDY P M S; YU C Y; BASTIANI C; HIGGS D; STAMATOYANNOPOULOS G; PAPAYANNOPOULOU T; SHEN C K J (Reprint)
AB We studied the functional interaction between human embryonic zeta2 globin promoter and the alpha globin regulatory element (HS-40) located 40 kb upstream of the zeta2 globin gene. It was shown by transient expression assay that HS-40 behaved as an authentic enhancer for high-level zeta2 globin promoter activity in K562 cells, an erythroid cell line of embryonic and/or fetal origin. Although sequences located between -559 and -88 of the zeta2 globin gene were dispensable for its expression on enhancerless plasmids, they were required for the **HS-40 enhancer**-mediated activity of the zeta2 globin promoter. Site-directed mutagenesis demonstrated that this **HS-40 enhancer**-zeta2 globin promoter interaction is mediated by the two GATA-1 factor binding motifs located at -230 and -104, respectively. The functional domains of HS-40 were also mapped. Bal 31 deletion mapping data

suggested that one GATA-1 motif, one GT motif, and two NF-E2/AP1 motifs together formed the functional core of HS-40 in the erythroid-specific activation of the zeta2 globin promoter. Site-directed mutagenesis further demonstrated that the enhancer function of one of the two NF-E2/AP1 motifs of HS-40 is mediated through its binding to NF-E2 but not AP1 transcription factor. Finally, we did genomic footprinting of the **HS-40 enhancer** region in K562 cells, adult nucleated erythroblasts, and different nonerythroid cells. All sequence motifs within the functional core of HS-40, as mapped by transient expression analysis, appeared to bind a nuclear factor(s) in living K562 cells but not in nonerythroid cells. On the other hand, only one of the apparently nonfunctional sequence motifs was bound with factors in vivo. In comparison to K562, nucleated erythroblasts from adult human bone marrow exhibited a similar but nonidentical pattern of nuclear factor binding in vivo at the HS-40 region. These data suggest that transcriptional activation of human embryonic zeta2 globin gene and the fetal/adult alpha globin genes is mediated by erythroid cell-specific and developmental stage-specific nuclear factor-DNA complexes which form at the enhancer (HS-40) and the globin promoters.

(FILE 'HOME' ENTERED AT 13:58:43 ON 13 JUN 2003)

FILE 'MEDLINE, AGRICOLA, CANCERLIT, SCISEARCH, CAPLUS, MEDICONF' ENTERED AT 13:59:23 ON 13 JUN 2003

```
L1          774 S ZETA(S)GLOBIN
L2          162 S L1 AND TRANSGENIC
L3            0 S L2 AND PIG
L4            1 S L1 AND PIG
L5            5 S L2 AND (HS-40 ENHANCER)
L6            5 DUP REM L5 (0 DUPLICATES REMOVED)
L7            5 SORT L6 PY
L8           45 S SHEN CHE-KUN JAMES/AU
                E SHEN CHE-KUN JAMES/AU
L9           45 S E1
L10           2 S L9 AND HS-40
```

=> d an ti so au ab pi l10 1-2

L10 ANSWER 1 OF 2 CAPLUS COPYRIGHT 2003 ACS

AN 2001:757858 CAPLUS

DN 135:314417

TI Vectors containing mutated **HS-40** enhancer of .zeta.-globin gene promoter and its regulation of transgene expression in transgenic mice

SO U.S., 7 pp., Division of U.S. Ser. No. 205,015, abandoned.
CODEN: USXXAM

IN **Shen, Che-Kun James**

AB The invention relates to a mutated **HS-40** enhancer of .zeta.-globin gene promoter, a 350-400 bp enhancer element located about 40 kb upstream of .zeta.-globin gene. **HS-40** is the major cis-acting regulatory element responsible for the developmental stage-and erythroid lineage-specific expression of the human .alpha.-like globin genes, the embryonic .zeta. and the adult .alpha.2/.alpha./1. The invention is based on the discovery that a single nucleotide change in the 3'NF-E2/AP1 element of the human **HS-40** enhancer, unlike the wild type **HS-40** enhancer, confers position-independent and copy no.-dependent expression on a transgene. In addn., the single nucleotide change allows expression of the gene in the cells of an adult mouse, an effect not seen for the wild type **HS -40** enhancer. Accordingly, the invention provides a viral expression vector (e.g., a retrovirus) expressing a transgene regulated by (1) a transcriptional start site; (2) a promoter (e.g., a tissue-specific promoter such as .zeta.-globin promoter) operably linked to the transcriptional start site; and (3) the above mutated **HS-40** enhancer operably linked to the promoter.

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	US 6303845	B1	20011016	US 2000-536094	20000324
	US 2002108134	A1	20020808	US 2001-977432	20011015

FILE 'HOME' ENTERED AT 14:11:23 ON 13 JUN 2003)

FILE 'MEDLINE, AGRICOLA, CANCERLIT, SCISEARCH, CAPLUS, MEDICONF' ENTERED AT 14:11:48 ON 13 JUN 2003

L1 27 S HS-40 ENHANCER
L2 10 DUP REM L1 (17 DUPLICATES REMOVED)
L3 7 S L2 AND MUT?
L4 7 SORT L3 PY

=> d an ti so au ab pi 3-6

L4 ANSWER 3 OF 7 MEDLINE
AN 95238333 MEDLINE
TI Functional roles of in vivo footprinted DNA motifs within an alpha-globin enhancer. Erythroid lineage and developmental stage specificities.
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (1995 Apr 14) 270 (15) 8501-5.
Journal code: 2985121R. ISSN: 0021-9258.
AU Zhang Q; Rombel I; Reddy G N; Gang J B; Shen C K
AB Transcriptional regulation of the human alpha-like globin genes, embryonic zeta 2 and adult alpha, during erythroid development is mediated by a distal enhancer, HS-40. Previous protein-DNA binding studies have shown that HS-40 consists of multiple nuclear factor binding motifs that are occupied in vivo in an erythroid lineage- and developmental stage-specific manner. We have systematically analyzed the functional roles of these factor binding motifs of HS-40 by site-directed **mutagenesis** and transient expression assay in erythroid cell cultures. Three of these **HS-40 enhancer** motifs, 5'NF-E2/AP1, GT II, and GATA-1(c), positively regulate the zeta 2-globin promoter activity in embryonic/fetal erythroid K562 cells and the adult alpha-globin promoter activity in adult erythroid MEL cells. On the other hand, the 3'NF-E2/AP1 motif is able to exert both positive and negative regulatory effects on the zeta 2-globin promoter activity in K562 cells, and this dual function appears to be modulated through differential binding of the ubiquitous AP1 factors and the erythroid-enriched NF-E2 factor. **Mutation** in the GATA-1(d) motif, which exhibits an adult erythroid-specific genomic footprint, decreases the **HS-40 enhancer** function in dimethyl sulfoxide-induced MEL cells but not in K562 cells. These studies have defined the regulatory roles of the different HS-40 motifs. The remarkable correlation between genomic footprinting data and the **mutagenesis** results also suggests that the erythroid lineage- and developmental stage-specific regulation of human alpha-like globin promoters is indeed modulated by stable binding of specific nuclear factors in vivo.

L4 ANSWER 4 OF 7 MEDLINE
AN 1999192436 MEDLINE
TI Development of viral vectors for gene therapy of beta-chain hemoglobinopathies: optimization of a gamma-globin gene expression cassette.
SO BLOOD, (1999 Apr 1) 93 (7) 2208-16.
Journal code: 7603509. ISSN: 0006-4971.
AU Li Q; Emery D W; Fernandez M; Han H; Stamatoyannopoulos G
AB Progress toward gene therapy of beta-chain hemoglobinopathies has been limited in part by poor expression of globin genes in virus vectors. To derive an optimal expression cassette, we systematically analyzed the sequence requirements and relative strengths of the Agamma- and beta-globin promoters, the activities of various erythroid-specific enhancers, and the importance of flanking and intronic sequences. Expression was analyzed by RNase protection after stable plasmid transfection of the murine erythroleukemia cell line, MEL585. Promoter truncation studies showed that the Agamma-globin promoter could be deleted to -159 without affecting expression, while deleting the beta-globin promoter to -127 actually increased expression compared with longer fragments. Expression from the optimal beta-globin gene promoter was consistently higher than that from the optimal Agamma-globin promoter, regardless of the enhancer used. Enhancers tested included a 2.5-kb composite of the beta-globin locus control region (termed a muLCR), a combination of the HS2 and HS3 core elements of the LCR, and the HS-40 core element of the alpha-globin locus. All three enhancers increased expression from the beta-globin gene to roughly the same extent, while the

HS-40 element was notably less effective with the Agamma-globin gene. However, the HS-40 element was able to efficiently enhance expression of a Agamma-globin gene linked to the beta-globin promoter. Inclusion of extended 3' sequences from either the beta-globin or the Agamma-globin genes had no significant effect on expression. A 714-bp internal deletion of Agamma-globin intron 2 unexpectedly increased expression more than twofold. With the combination of a -127 beta-globin promoter, an Agamma-globin gene with the internal deletion of intron 2, and a single copy of the **HS-40 enhancer**, gamma-globin expression averaged 166% of murine alpha-globin mRNA per copy in six pools and 105% in nine clones. When placed in a retrovirus vector, this cassette was also expressed at high levels in MEL585 cells (averaging 75% of murine alpha-globin mRNA per copy) without reducing virus titers. However, recombined provirus or aberrant splicing was observed in 5 of 12 clones, indicating a significant degree of genetic instability. Taken together, these data demonstrate the development of an optimal expression cassette for gamma-globin capable of efficient expression in a retrovirus vector and form the basis for further refinement of vectors containing this cassette.

L4 ANSWER 5 OF 7 MEDLINE
AN 2000153760 MEDLINE
TI Loading of DNA-binding factors to an erythroid enhancer.
SO MOLECULAR AND CELLULAR BIOLOGY, (2000 Mar) 20 (6) 1993-2003.
Journal code: 8109087. ISSN: 0270-7306.
AU Wen S C; Roder K; Hu K Y; Rombel I; Gavva N R; Daftari P; Kuo Y Y; Wang C; Shen C K
AB The **HS-40 enhancer** is the major cis-acting regulatory element responsible for the developmental stage- and erythroid lineage-specific expression of the human alpha-like globin genes, the embryonic zeta and the adult alpha2/alpha/1. A model has been proposed in which competitive factor binding at one of the HS-40 motifs, 3'-NA, modulates the capability of HS-40 to activate the embryonic zeta-globin promoter. Furthermore, this modulation was thought to be mediated through configurational changes of the HS-40 enhanceosome during development. In this study, we have further investigated the molecular basis of this model. First, human erythroid K562 cells stably integrated with various HS-40 **mutants** cis linked to a human alpha-globin promoter-growth hormone hybrid gene were analyzed by genomic footprinting and expression analysis. By the assay, we demonstrate that factors bound at different motifs of HS-40 indeed act in concert to build a fully functional enhanceosome. Thus, modification of factor binding at a single motif could drastically change the configuration and function of the HS-40 enhanceosome. Second, a specific 1-bp, GC-->TA **mutation** in the 3'-NA motif of HS-40, 3'-NA(II), has been shown previously to cause significant derepression of the embryonic zeta-globin promoter activity in erythroid cells. This derepression was hypothesized to be regulated through competitive binding of different nuclear factors, in particular AP1 and NF-E2, to the 3'-NA motif. By gel mobility shift and transient cotransfection assays, we now show that 3'-NA(II) **mutation** completely abolishes the binding of small MafK homodimer. Surprisingly, NF-E2 as well as AP1 can still bind to the 3'-NA(II) sequence. The association constants of both NF-E2 and AP1 are similar to their interactions with the wild-type 3'-NA motif. However, the 3'-NA(II) **mutation** causes an approximately twofold reduction of the binding affinity of NF-E2 factor to the 3'-NA motif. This reduction of affinity could be accounted for by a twofold-higher rate of dissociation of the NF-E2-3'-NA(II) complex. Finally, we show by chromatin immunoprecipitation experiments that only binding of NF-E2, not AP1, could be detected in vivo in K562 cells around the HS-40 region. These data exclude a role for AP1 in the developmental regulation of the human alpha-globin locus via the 3'-NA motif of HS-40 in embryonic/fetal erythroid cells. Furthermore, extrapolation of the in vitro binding studies suggests that factors other than NF-E2, such as the small Maf homodimers, are likely involved in the regulation of the HS-40 function in vivo.

L4 ANSWER 6 OF 7 CAPLUS COPYRIGHT 2003 ACS
AN 2001:757858 CAPLUS
DN 135:314417

TI Vectors containing **mutated HS-40** **enhancer** of .zeta.-globin gene promoter and its regulation of transgene expression in transgenic mice

SO U.S., 7 pp., Division of U.S. Ser. No. 205,015, abandoned.
CODEN: USXXAM

IN Shen, Che-Kun James

AB The invention relates to a **mutated HS-40** **enhancer** of .zeta.-globin gene promoter, a 350-400 bp enhancer element located about 40 kb upstream of .zeta.-globin gene. HS-40 is the major cis-acting regulatory element responsible for the developmental stage-and erythroid lineage-specific expression of the human .alpha.-like globin genes, the embryonic .zeta. and the adult .alpha.2/.alpha./1. The invention is based on the discovery that a single nucleotide change in the 3'NF-E2/AP1 element of the human **HS-40** **enhancer**, unlike the wild type **HS-40** **enhancer**, confers position-independent and copy no.-dependent expression on a transgene. In addn., the single nucleotide change allows expression of the gene in the cells of an adult mouse, an effect not seen for the wild type **HS-40** **enhancer**. Accordingly, the invention provides a viral expression vector (e.g., a retrovirus) expressing a transgene regulated by (1) a transcriptional start site; (2) a promoter (e.g., a tissue-specific promoter such as .zeta.-globin promoter) operably linked to the transcriptional start site; and (3) the above **mutated HS-40** **enhancer** operably linked to the promoter.

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI US 6303845	B1	20011016	US 2000-536094	20000324
US 2002108134	A1	20020808	US 2001-977432	20011015

=>

```
L1            42 S HS-40 ENHANCER
L2            11 DUP REM L1 (31 DUPLICATES REMOVED)
L3          1326 S ZETA(S)GLOBIN
L4           237 S L3 AND TRANSGENIC
L5            11 SORT L2 PY
L6            35 S L4 AND HS-40
L7            15 DUP REM L6 (20 DUPLICATES REMOVED)
L8            25 S L7 OR L2
L9            20 DUP REM L8 (5 DUPLICATES REMOVED)
L10           20 SORT L9 PY

=> d an ti so au ab pi 19 16 15 11 10 9 8 4 3 2
```

L10 ANSWER 19 OF 20 CAPLUS COPYRIGHT 2002 ACS
AN 2001:757858 CAPLUS
DN 135:314417
TI Vectors containing mutated **HS-40 enhancer** of **.zeta.-globin** gene promoter and its regulation of transgene expression in **transgenic** mice
SO U.S., 7 pp., Division of U.S. Ser. No. 205,015, abandoned.
CODEN: USXXAM
IN Shen, Che-Kun James
AB The invention relates to a mutated **HS-40 enhancer** of **.zeta.-globin** gene promoter, a 350-400 bp enhancer element located about 40 kb upstream of **.zeta.-globin** gene. **HS-40** is the major cis-acting regulatory element responsible for the developmental stage-and erythroid lineage-specific expression of the human .alpha.-like **globin** genes, the embryonic **.zeta.** and the adult .alpha.2/.alpha./1. The invention is based on the discovery that a single nucleotide change in the 3'NF-E2/AP1 element of the human **HS-40 enhancer**, unlike the wild type **HS-40 enhancer**, confers position-independent and copy no.-dependent expression on a transgene. In addn., the single nucleotide change allows expression of the gene in the cells of an adult mouse, an effect not seen for the wild type **HS-40 enhancer**. Accordingly, the invention provides a viral expression vector (e.g., a retrovirus) expressing a transgene regulated by (1) a transcriptional start site; (2) a promoter (e.g., a tissue-specific promoter such as **.zeta.-globin** promoter) operably linked to the transcriptional start site; and (3) the above mutated **HS-40 enhancer** operably linked to the promoter.

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI US 6303845	B1	20011016	US 2000-536094	20000324
US 2002108134	A1	20020808	US 2001-977432	20011015

L10 ANSWER 16 OF 20 SCISEARCH COPYRIGHT 2002 ISI (R)
AN 2000:647407 SCISEARCH
TI Non-erythroid genes inserted on either side of human **HS-40** impair the activation of its natural alpha-globin gene targets without being themselves preferentially activated
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (18 AUG 2000) Vol. 275, No. 33, pp. 25831-25839.
Publisher: AMER SOC BIOCHEMISTRY MOLECULAR BIOLOGY INC, 9650 ROCKVILLE PIKE, BETHESDA, MD 20814.
ISSN: 0021-9258.
AU Esperet C; Sabatier S; Deville M A; Ouazana R; Bouhassira E E; Godet J; Morle F; Bernet A (Reprint)
AB The human cu-**globin** gene complex includes three functional **globin** genes (5'-**zeta** 2-alpha 2-alpha 1-3') regulated by a common positive regulatory element named **HS-40** displaying strong erythroid-specific enhancer activity. How this enhancer activity can be shared between different promoters present at different positions in the same complex is poorly understood. To address this question, we used homologous recombination to target the insertion of marker genes driven by cytomegalovirus or long terminal repeat promoters in both possible orientations either upstream or downstream from the **HS-40** region into the single human alpha-**globin**

gene locus present in hybrid mouse erythroleukemia cells. We also used CRE recombinase-mediated cassette exchange to target the insertion of a tagged alpha-**globin** gene at the same position downstream from **HS-40**. All these insertions led to a similar decrease in the **HS-40**-dependent transcription of downstream human alpha-**globin** genes in differentiated cells. Interestingly, this decrease is associated with the strong activation of the proximal newly inserted alpha-**globin** gene, whereas in marked contrast, the transcription of the non-erythroid marker genes remains insensitive to **HS-40**. Taken together, these results indicate that the enhancer activity of **HS-40** can be trapped by non-erythroid promoters in both upstream and downstream directions without necessarily leading to their own activation.

L10 ANSWER 15 OF 20 MEDLINE
AN 2000153760 MEDLINE
TI Loading of DNA-binding factors to an erythroid enhancer.
SO MOLECULAR AND CELLULAR BIOLOGY, (2000 Mar) 20 (6) 1993-2003.
Journal code: 8109087. ISSN: 0270-7306.
AU Wen S C; Roder K; Hu K Y; Rombel I; Gavva N R; Daftari P; Kuo Y Y; Wang C; Shen C K
AB The **HS-40 enhancer** is the major cis-acting regulatory element responsible for the developmental stage- and erythroid lineage-specific expression of the human alpha-like globin genes, the embryonic zeta and the adult alpha2/alpha/1. A model has been proposed in which competitive factor binding at one of the HS-40 motifs, 3'-NA, modulates the capability of HS-40 to activate the embryonic zeta-globin promoter. Furthermore, this modulation was thought to be mediated through configurational changes of the HS-40 enhanceosome during development. In this study, we have further investigated the molecular basis of this model. First, human erythroid K562 cells stably integrated with various HS-40 mutants cis linked to a human alpha-globin promoter-growth hormone hybrid gene were analyzed by genomic footprinting and expression analysis. By the assay, we demonstrate that factors bound at different motifs of HS-40 indeed act in concert to build a fully functional enhanceosome. Thus, modification of factor binding at a single motif could drastically change the configuration and function of the HS-40 enhanceosome. Second, a specific 1-bp, GC-->TA mutation in the 3'-NA motif of HS-40, 3'-NA(II), has been shown previously to cause significant derepression of the embryonic zeta-globin promoter activity in erythroid cells. This derepression was hypothesized to be regulated through competitive binding of different nuclear factors, in particular AP1 and NF-E2, to the 3'-NA motif. By gel mobility shift and transient cotransfection assays, we now show that 3'-NA(II) mutation completely abolishes the binding of small MafK homodimer. Surprisingly, NF-E2 as well as AP1 can still bind to the 3'-NA(II) sequence. The association constants of both NF-E2 and AP1 are similar to their interactions with the wild-type 3'-NA motif. However, the 3'-NA(II) mutation causes an approximately twofold reduction of the binding affinity of NF-E2 factor to the 3'-NA motif. This reduction of affinity could be accounted for by a twofold-higher rate of dissociation of the NF-E2-3'-NA(II) complex. Finally, we show by chromatin immunoprecipitation experiments that only binding of NF-E2, not AP1, could be detected in vivo in K562 cells around the HS-40 region. These data exclude a role for AP1 in the developmental regulation of the human alpha-globin locus via the 3'-NA motif of HS-40 in embryonic/fetal erythroid cells. Furthermore, extrapolation of the in vitro binding studies suggests that factors other than NF-E2, such as the small Maf homodimers, are likely involved in the regulation of the HS-40 function in vivo.

L10 ANSWER 11 OF 20 MEDLINE
AN 1999061925 MEDLINE
TI Derepression of human embryonic **zeta-globin** promoter by a locus-control region sequence.
SO PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1998 Dec 8) 95 (25) 14669-74.
Journal code: 7505876. ISSN: 0027-8424.
AU Huang B L; Fan-Chiang I R; Wen S C; Koo H C; Kao W Y; Gavva N R; Shen C K
AB A multiple protein-DNA complex formed at a human alpha-**globin** locus-specific regulatory element, **HS-40**, confers

appropriate developmental expression pattern on human embryonic **zeta-globin** promoter activity in humans and **transgenic** mice. We show here that introduction of a 1-bp mutation in an NF-E2/AP1 sequence motif converts **HS-40** into an erythroid-specific locus-control region. Cis-linkage with this locus-control region, in contrast to the wild-type **HS-40**, allows erythroid lineage-specific derepression of the silenced human **zeta-globin** promoter in fetal and adult **transgenic** mice. Furthermore, **zeta-globin** promoter activities in adult mice increase in proportion to the number of integrated DNA fragments even at 19 copies/genome. The mutant **HS-40** in conjunction with human **zeta-globin** promoter thus can be used to direct position-independent and copy number-dependent expression of transgenes in adult erythroid cells. The data also supports a model in which competitive DNA binding of different members of the NF-E2/AP1 transcription factor family modulates the developmental stage specificity of an erythroid enhancer. Feasibility to reswitch on embryonic/fetal **globin** genes through the manipulation of nuclear factor binding at a single regulatory DNA motif is discussed.

L10 ANSWER 10 OF 20 SCISEARCH COPYRIGHT 2002 ISI (R)
AN 97:168369 SCISEARCH
TI A globin enhancer acts by increasing the proportion of erythrocytes expressing a linked transgene
SO MOLECULAR AND CELLULAR BIOLOGY, (MAR 1997) Vol. 17, No. 3, pp. 1607-1614. Publisher: AMER SOC MICROBIOLOGY, 1325 MASSACHUSETTS AVENUE, NW, WASHINGTON, DC 20005-4171.
ISSN: 0270-7306.
AU Sutherland H G E; Martin D I K; Whitelaw E (Reprint)
AB Enhancer elements have been shown to affect the probability of a gene establishing an active transcriptional state and suppress the silencing of reporter genes in cell lines, but their effect in transgenic mice has been obscured by the use of assays that do not assess expression on a cell-by-cell basis. We have examined the effect of a globin enhancer on the variegation of lacZ expression in erythrocytes of transgenic mice. Mice carrying lacZ driven by the alpha-globin promoter exhibit beta-galactosidase (beta-Gal) expression in only a very small proportion of embryonic erythrocytes, When the transgenic construct also contains the alpha **HS-40 enhancer**, which controls expression of the alpha-globin gene, expression is seen in a high proportion of embryonic erythrocytes, although there are variations between transgenic lines which can be attributed to different sites of integration. Analysis of beta-Gal expression levels suggests that expressing cells in lines carrying only the alpha-globin promoter express as much beta-Gal as those in which the transgene also contains alpha HS-40. A marked decline in transgene expression occurs as mice age, which is mainly due to a decrease in the proportion of cells expressing the transgene. Thus, a globin enhancer can act to suppress variegation of a linked transgene; this result is consistent with a model in which enhancers act to establish and maintain an active domain without directly affecting the transcriptional rate.

L10 ANSWER 9 OF 20 SCISEARCH COPYRIGHT 2002 ISI (R)
AN 97:562553 SCISEARCH
TI Analysis of enhancer function of the **HS-40** core sequence of the human alpha-globin cluster
SO NUCLEIC ACIDS RESEARCH, (15 JUL 1997) Vol. 25, No. 14, pp. 2917-2922. Publisher: OXFORD UNIV PRESS, GREAT CLARENDON ST, OXFORD, ENGLAND OX2 6DP.
ISSN: 0305-1048.
AU Chen H L; Lowrey C H; Stamatoyannopoulos G (Reprint)
AB **HS-40** is the major regulatory element of the human alpha-**globin** locus, located 40 kb upstream of the **zeta-globin** gene, To test for potential interactions between **HS-40** and the beta- or the gamma-**globin** gene promoters in stable transfection assays, the **HS-40** core sequence was cloned upstream of either the beta promoter or the gamma promoter driving the neomycin phosphotransferase gene and enhancer activity was measured using a colony assay, In K562 or in MEL cells, enhancer activity of **HS-40** was higher than that of the

individual core sequences of the DNase I hypersensitive sites (HS) of the beta-**globin** locus control region (LCR), and similar to 60% of the enhancer activity of a 2.5 kb mu LCR, which contains the core elements of DNase I hypersensitive sites 1-4. In contrast to the synergistic interaction between the DNase I hypersensitive sites of beta locus LCR, combination of **HS-40** with these DNase I hypersensitive sites failed to display cooperativity in K562 cells and inhibited enhancer function in MEL cells, Inhibition of enhancer function was also observed when two copies of the **HS-40** were arranged tandemly, We conclude that the core element of **HS-40** (i) is a powerful enhancer of gamma- and beta-**globin** gene expression, (ii) in contrast to other classical enhancers, acts best as a single copy, (iii) does not cooperate with the regulatory elements of the beta-**globin** locus control region.

L10 ANSWER 8 OF 20 MEDLINE
AN 97086677 MEDLINE
TI Proximal promoter elements of the human **zeta-globin** gene confer embryonic-specific expression on a linked reporter gene in **transgenic** mice.
SO NUCLEIC ACIDS RESEARCH, (1996 Nov 1) 24 (21) 4158-64.
Journal code: 0411011. ISSN: 0305-1048.
AU Pondel M D; Sharpe J A; Clark S; Pearson L; Wood W G; Proudfoot N J
AB We have investigated the transcriptional regulation of the human embryonic **zeta-globin** gene promoter. First, we examined the effect that deletion of sequences 5' to **zeta-globin**'s CCAAT box have on **zeta**-promoter activity in erythroid cell lines. Deletions of sequences between -116 and -556 (cap = 0) had little effect while further deletion to -84 reduced **zeta**-promoter activity by only 2-3-fold in both transiently and stably transfected erythroid cells. Constructs containing 67, 84 and 556 bp of **zeta-globin** 5' flanking region linked to a beta-galactosidase reporter gene (lacZ) and hypersensitive site -40 (**HS-40**) of the human alpha-**globin** gene cluster were then employed for the generation of **transgenic** mice. LacZ expression from all constructs, including a 67 bp **zeta-globin** promoter, was erythroid-specific and most active between 8.5 and 10.5 days post-fertilisation. By 16.5 days gestation, lacZ expression dropped 40-100-fold. These results suggest that embryonic-specific activation of the human **zeta-globin** promoter is conferred by a 67 bp **zeta**-promoter fragment containing only a CCAAT and TATA box.

L10 ANSWER 4 OF 20 MEDLINE
AN 95023182 MEDLINE
TI Analysis of a 70 kb segment of DNA containing the human **zeta** and alpha-**globin** genes linked to their regulatory element (**HS-40**) in **transgenic** mice.
SO NUCLEIC ACIDS RESEARCH, (1994 Oct 11) 22 (20) 4139-47.
Journal code: 0411011. ISSN: 0305-1048.
AU Gourdon G; Sharpe J A; Wells D; Wood W G; Higgs D R
AB We have ligated two cosmids through an oligonucleotide linker to produce a single fragment spanning 70 kb of the human alpha-**globin** cluster, in which the alpha-like **globin** genes (**zeta** 2, alpha 2 and alpha 1), their regulatory element (**HS-40**) and erythroid-specific DNase I hypersensitive sites accurately retain their normal genomic organization. The **zeta** (embryonic) and alpha (embryonic, fetal and adult) **globin** genes were expressed in all 17 **transgenic** embryos. Similarly, all fetal and adult mice from seven **transgenic** lines that contained one or more copies of the fragment, produced up to 66% of the level of endogenous mouse alpha-**globin** mRNA. However, as for smaller constructs containing these elements, human alpha-**globin** expression was not copy number dependent and decreased by 1.5-9.0 fold during development. These findings suggest that either it is not possible to obtain full regulation of human alpha-**globin** expression in **transgenic** mice or, more likely, that additional alpha-**globin** regulatory elements lie beyond the 70 kb segment of DNA analysed.

L10 ANSWER 3 OF 20 MEDLINE

AN 93204975 MEDLINE
TI Transcriptional activation of human zeta 2 globin promoter by the alpha globin regulatory element (HS-40): functional role of specific nuclear factor-DNA complexes.
SO MOLECULAR AND CELLULAR BIOLOGY, (1993 Apr) 13 (4) 2298-308.
Journal code: 8109087. ISSN: 0270-7306.
AU Zhang Q; Reddy P M; Yu C Y; Bastiani C; Higgs D; Stamatoyannopoulos G; Papayannopoulou T; Shen C K
AB We studied the functional interaction between human embryonic zeta 2 globin promoter and the alpha globin regulatory element (HS-40) located 40 kb upstream of the zeta 2 globin gene. It was shown by transient expression assay that HS-40 behaved as an authentic enhancer for high-level zeta 2 globin promoter activity in K562 cells, an erythroid cell line of embryonic and/or fetal origin. Although sequences located between -559 and -88 of the zeta 2 globin gene were dispensable for its expression on enhancerless plasmids, they were required for the **HS -40 enhancer**-mediated activity of the zeta 2 globin promoter. Site-directed mutagenesis demonstrated that this **HS-40 enhancer**-zeta 2 globin promoter interaction is mediated by the two GATA-1 factor binding motifs located at -230 and -104, respectively. The functional domains of HS-40 were also mapped. Bal 31 deletion mapping data suggested that one GATA-1 motif, one GT motif, and two NF-E2/AP1 motifs together formed the functional core of HS-40 in the erythroid-specific activation of the zeta 2 globin promoter. Site-directed mutagenesis further demonstrated that the enhancer function of one of the two NF-E2/AP1 motifs of HS-40 is mediated through its binding to NF-E2 but not AP1 transcription factor. Finally, we did genomic footprinting of the **HS-40 enhancer** region in K562 cells, adult nucleated erythroblasts, and different nonerythroid cells. All sequence motifs within the functional core of HS-40, as mapped by transient expression analysis, appeared to bind a nuclear factor(s) in living K562 cells but not in nonerythroid cells. On the other hand, only one of the apparently nonfunctional sequence motifs was bound with factors in vivo. In comparison to K562, nucleated erythroblasts from adult human bone marrow exhibited a similar but nonidentical pattern of nuclear factor binding in vivo at the HS-40 region. These data suggest that transcriptional activation of human embryonic zeta 2 globin gene and the fetal/adult alpha globin genes is mediated by erythroid cell-specific and developmental stage-specific nuclear factor-DNA complexes which form at the enhancer (HS-40) and the globin promoters.

L10 ANSWER 2 OF 20 MEDLINE
AN 94068584 MEDLINE
TI Analysis of the human alpha-globin gene cluster in **transgenic** mice.
SO PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1993 Dec 1) 90 (23) 11262-6.
Journal code: 7505876. ISSN: 0027-8424.
AU Sharpe J A; Wells D J; Whitelaw E; Vyas P; Higgs D R; Wood W G
AB A 350-bp segment of DNA associated with an erythroid-specific DNase I-hypersensitive site (**HS-40**), upstream of the alpha-**globin** gene cluster, has been identified as the major tissue-specific regulator of the alpha-**globin** genes. However, this element does not direct copy number-dependent or developmentally stable expression of the human genes in **transgenic** mice. To determine whether additional upstream hypersensitive sites could provide more complete regulation of alpha gene expression we have studied 17 lines of **transgenic** mice bearing various DNA fragments containing HSs -33, -10, -8, and -4, in addition to **HS -40**. Position-independent, high-level expression of the human **zeta**- and alpha-**globin** genes was consistently observed in embryonic erythroid cells. However, the additional HSs did not confer copy-number dependence, alter the level of expression, or prevent the variable down-regulation of expression in adults. These results suggest that the region upstream of the human alpha-**globin** genes is not equivalent to that upstream of the beta locus and that although the two clusters are coordinately expressed, there may be differences in their regulation.